Ultramicrotechnique for Enzymatic Hydrolysis of Sugars Prior to Chromatogram Analysis

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Previously described methods for the enzymatic hydrolysis of sugars on filter paper are not applicable when the hydrolysis is a slow process. An ultramicromethod is given in which 20 to 30 γ of sugar can be hydrolyzed in melting point tubes. The hydrolysis products are then analyzed on a papergram.

THE qualitative analysis of carbohydrates is often greatly facilitated by examination of the products obtained through enzyme action. The activity or inactivity of specific yeasts toward certain sugars has been used for qualitative and quantitative analysis (*1*, *2*, *6*, *8*). Yeast fermentation prior to papergram analysis has also been employed (*3*, *6*). Several procedures have been published for the enzymic treatment of sugars followed by resolution and identification of the components of the resulting mixture by means of paper chromatography. Williams and Bevenue (*10*) suggested superimposing a drop of enzyme solution on a drop of sugar solution already placed on the starting line of a chromatogram. After a relatively short time, the spot was allowed to dry and the chromatogram was developed. From the products identified a more complete picture of the unknown compound could be obtained.

This technique, using 20 to 30 γ of sugar, is an invaluable tool and works extremely well with invertase for such oligosaccharides as sucrose and raffinose. However, during a study of the carbohydrate content of maple sap it was found that the melibiase hydrolysis of melibiose is a much slower process and that it was impossible to obtain complete hydrolysis on paper, as it was impractical to wet the spot for the time required. Since the quantity of samples available for certain identification experiments was extremely limited, an ultramicromethod has been developed based upon techniques used by Schneider (*7*) for qualitative organic analysis. In this method 0.01-ml. samples of solutions, containing 20 to 30 γ of sugar, can be enzymatically hydrolyzed in melting point tubes and the entire sample can be deposited on paper for resolution of the resulting mixture.

EQUIPMENT AND MATERIALS

The usual paper chromatographic equipment.
Melting point tubes, soft glass, 1 to 2 by 100 mm.
Small centrifuge.
Microburner.
Carbohydrate solution (5 mg. per ml.) preserved with toluene.
Enzyme solutions (10 mg. per ml.) preserved with toluene.

METHOD

Figures 1 and 2 show the steps followed from the treatment of the sample through the application of the material to the paper. The drawings labeled *a* to *h* (Figure 1) indicate the steps up to, and including, the incubation period. The drawings labeled *i* to *o* (Figure 2) indicate the subsequent steps up to, and including, placement of the hydrolyzed solutions on the filter paper.

Place 1 drop of sugar solution on a microscope slide or in a small test tube. Place a drop of the enzyme solution at the other end of the slide or in another tube. Dip one end of a melting point tube into the sugar solution and allow a volume of 0.01 ml. (about 5 mm., depending upon the tube diameter) to be drawn into the tube by capillarity, *a*. Remove the tube and hold it at such an angle as to allow the column of solution to flow to about 1 cm.

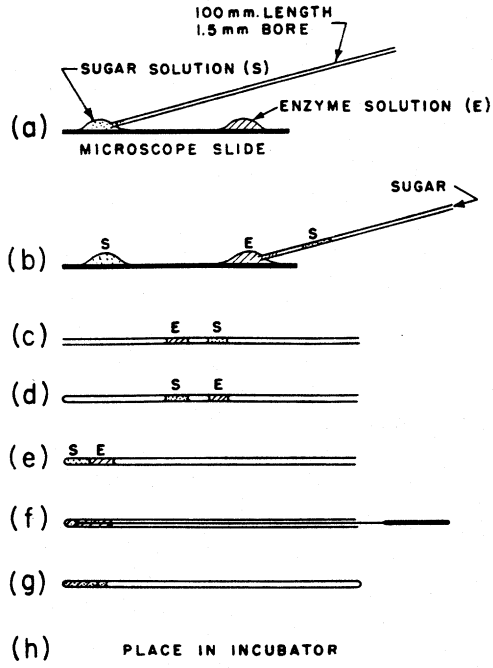

Figure 1. Steps Required in Preparing Sample for Incubation

from the opposite end. Dip this clean end of the melting point tube into the enzyme solution until the same volume is taken up, *b*. Remove the tube and, by holding at the proper angle, cause the two columns of solution to flow to the tube center, *c*. Wipe off the ends of the tube and seal the carbohydrate end of the tube in a microflame, *d*. Cool and then centrifuge, moving both solutions together into the sealed end, *e*. Mix the two solutions by use of a glass thread drawn from a fine glass rod, *f*. Seal the open end of the tube in a microflame, *g*. Centrifuge the contents back and forth several times to ensure thorough mixing. (Centrifuging alone does not always give adequate mixing.) Place the sealed tube in an incubator for the predetermined period of time, *h*.

After the incubation period, break the empty end of the tube, place the tube in a microflame, *i*, and draw out to a fine threadlike point, *j*. Make a small bead on the threadlike tip to add strength, *k*. Place the tube in a centrifuge and move the contents to the drawn out end of the tube, *l*. Scratch the tube about 1 cm. from the opposite end and break off, *m*. Using a pair of tweezers, break off the bead on the fine tip, *n*. Apply the tube contents to the chromatogram using the tube as a pipet, *o*. Multiple drops can be deposited on one spot if a warm air stream is applied to the paper so as to dry it between applications. Best results are obtained if the solution spreads to a diameter no greater than 3 to 4 mm. Place the paper in a chromatographic tank and develop. Spray with indicating solution to bring out the positions of the sugars.

Table I shows the results obtained using this method for the enzymatic hydrolysis of several carbohydrates. The products indicated by the chromatogram analyses are presented along with the R_g values (referred to glucose). The developing solvent employed was isopropyl alcohol–*n*-butyl alcohol–water (7:1:2, single phase) as recommended by Gross and Albon (*4*). The indicating spray was benzidine citrate as developed by White and Maher (*9*):

Solution A, 0.14 gram of benzidine per 100 ml. of *n*-butyl alcohol; Solution B, 50% citric acid monohydrate in water.

Just prior to use, mix 3 parts of solution *A* with one part of solution B. Spray and heat at 105° C. for 5 minutes.

DISCUSSION

As diastase required 96 hours to hydrolyze maltose to glucose and the same enzyme did not produce complete hydrolysis of starch in 168 hours, it is evident why it was impossible to obtain complete hydrolysis directly on filter paper. The technique described herein does produce complete hydrolysis and employs the same ultramicrosamples. The procedure has, therefore, made possible studies of optimum temperature, pH, and sugar-enzyme concentrations on limited samples. By placing the hydrolytic tube in boiling water the enzyme can be inactivated and the hydrolysis stopped. The 4-hour maltose sample in Table I was inactivated in this manner. Chromatogram analysis of the products showed, in addition to the expected maltose and glucose, two other oligosaccharides as demonstrated by Pan *et al.* (*5*).

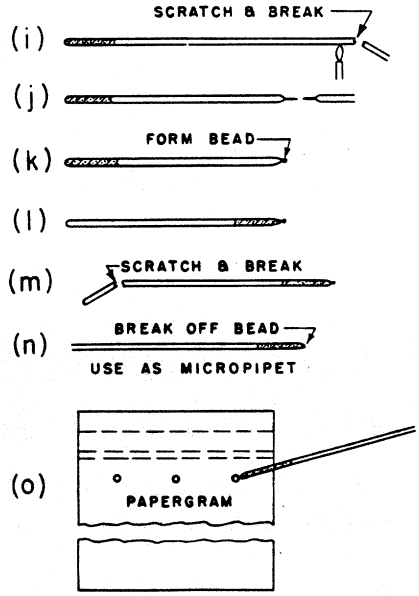

Figure 2. Steps Required in Preparing Tube for Placement of Hydrolyzed Sample on Paper Prior to Development

Table I. Chromatogram Analyses[a] of Carbohydrates Enzymatically Hydrolyzed by Ultramicromethod

Concentration of carbohydrate solution, 5 mg. per ml.
Volume for hydrolysis, 0.01 ml.
Temperature of incubation, 37° C.

Carbohydrate	Enzyme	Time of Hydrolysis, Hr.	Resulting Carbohydrate with R_g[b] Value
Sucrose	Invertase[c]	4	Glucose (1.01), fructose (1.15)
Raffinose	Invertase	24	Melibiose (0.37), fructose (1.14)
Raffinose	Invertase and melibiase	72	Galactose (0.88), glucose (1.00), fructose (1.15)
Melibiose	Invertase	24	Melibiose (0.37)
Melibiose	Invertase and melibiase	72	Galactose (0.88), glucose (1.00)
Maltose	Diastase	4	Trace unknowns (0.09, 0.23), maltose (0.50), glucose (1.01)
Maltose	Diastase	48	Trace unknown (0.23), maltose (0.50), glucose (1.00)
Maltose	Diastase	96	Glucose (1.00)
Starch	Diastase	24	Unknowns (0.00, 0.09, 0.23), maltose (0.50), glucose (1.01)
Starch	Diastase	72	Trace unknowns (0.00, 0.09, 0.23), maltose (0.50), glucose (1.00)
Starch	Diastase	168	Slight traces of unknowns and maltose, glucose (1.00)

[a] Developing solvent = Isopropyl alcohol: *n*-butyl alcohol: water (7:1:2 single phase) (*4*).
[b] R_g values agree closely with known carbohydrates run simultaneously.
[c] All enzyme concentrations arbitrarily set at 10 mg. per ml.

The procedure, with slight modification, appears to be applicable to enzyme systems other than those reported.

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